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DETERMINATION OF 1,4- AND 1,5-BENZODIAZEPINES IN URINE USING A COMPUTERIZED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC TECHNIQUE*

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SUMMARY

A method for the determination of benzodiazepines and their main metabolites in urine after acid hydrolysis is described. The extract is analyzed by computerized gas chromatography—mass spectrometry. An on-line computer allows rapid detection using mass fragmentography with the masses *m/e* 211, 230, 241, 244, 249, 262, 276, and 285. The mass fragmentogram and the underlying mass spectra of the hydrolysis products (benzophenones and analogues) are documented.

INTRODUCTION

In the last few years ever more benzodiazepines have been put on to the market. Because of their immense application these drugs are encountered very frequently in clinical and forensic toxicology. Screening for these drugs by their determination in the urine after acid hydrolysis to decompose conjugates is recommended. Thereby the benzodiazepine molecules are also decomposed [1]. Decomposition of the conjugates by enzymatic hydrolysis has the advantage that it leaves the benzodiazepine molecules intact, but typically takes 12 h and so does not lend itself to a rapid screening procedure.

Identification of some of the acid hydrolysis products (benzophenones and analogues) employing thin-layer chromatography [2], gas—liquid chromatography [2,3] or high-performance liquid chromatography [4] has been described. In the review article of Hailey [5] several detection methods and

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further references are given. However, none of these methods allows the rapid and exact differentiation that is important in clinical or forensic estimation because the various benzodiazepines have very different pharmacological potencies. These demands are met by the computerized gas chromatographic—mass spectrometric (GC—MS) technique described below.

EXPERIMENTAL

Apparatus

A Varian Aerograph gas chromatograph series 1400 combined with a Varian mass spectrometer Type 331 A, a Varian data system 111 MS and a Tektronix storage display unit Type 611 was used. The GC conditions were as follows. Column: nickel-capillary 60 cm × 1 mm I.D., packed with Chromosorb W AW DMCS 80—100 mesh re-silanized with dichlorodimethylsilane and coated with 20% UCC-W (Hewlett-Packard). (OV-1 can also be used.) Column temperature: programmed from 100 to 310°C at 20°C/min. Injector port temperature: 270°C. Carrier gas: helium, flow-rate 7 ml/min.

The MS conditions were as follows: ionization energy, 90 eV; ion-source temperature, 200°C. The technique of open coupling was used. About 2 ml/min of gas were dosed by an SGE micro-needle-valve and an SGE shut-off valve (Scientific Glass Engineering, Ringwood, Australia) and introduced into the ion source by a nickel capillary (0.25 mm I.D. heated at 270°C). Details will be published later [6].

For the exact measurement of retention indices a Varian Aerograph gas chromatograph series 3700 was used. The column effluent went to a flame-ionization detector (FID) and a nitrogen-sensitive FID (N-FID) after a 1:1 split by a splitter made from nickel tubing. The column was a nickel tube, 60 cm × 2 mm I.D., packed as for GC—MS but with only 10% stationary phase. The column and injector temperatures were as for GC—MS, the temperature of the detectors was 270°C. Carrier gas was nitrogen at a flow-rate of 30 ml/min.

Hydrolysis and extraction procedure

Ten millilitres of urine are refluxed with 3 ml of hydrochloric acid (37%) for 15 min, then made basic with about 3 g of potassium hydroxide pellets and mixed with 10 ml of 30% aqueous ammonium sulfate to obtain a pH of between 8 and 9. The sample is then extracted twice with 10 ml of diethyl ether. After phase separation by centrifugation the combined ether extracts are evaporated to dryness under vacuum. The residue is redissolved in 0.1 ml of methanol and 1—4 μl of this solution are injected into the gas chromatograph.

Gas chromatographic—mass spectrometric analysis

Mass spectra are recorded at a speed of 6 sec/decade and stored on computer tape during the temperature-programmed GC analysis. Scanning at this relatively slow rate ensures at least two spectra for each GC peak whilst avoiding excessive data accumulation. The identity of positive signals in the reconstructed mass fragmentogram is established by a comparison of the entire mass spectra with those of standards.

RESULTS AND DISCUSSION

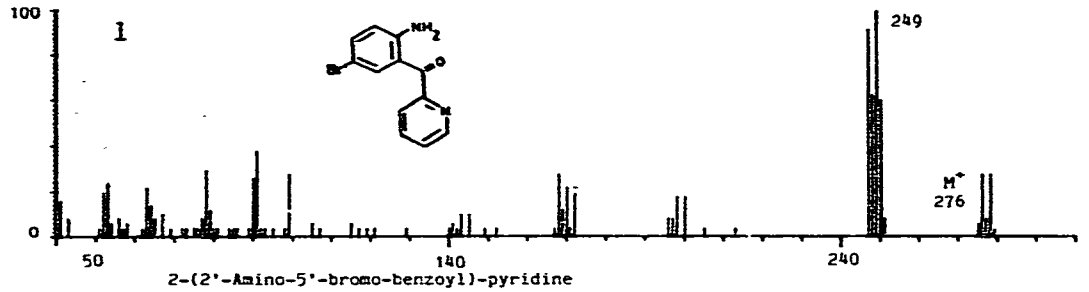
The results of our investigations are shown in Table I. The mass fragmentogram with the eight proposed masses shown in Table I allows the detection of the hydrolysis products (benzophenones and analogues) of sixteen important benzodiazepines as themselves or their metabolites. But not all metabolites are listed; only those detectable by the mass fragmentogram and necessary for the identification of the drugs.

TABLE I

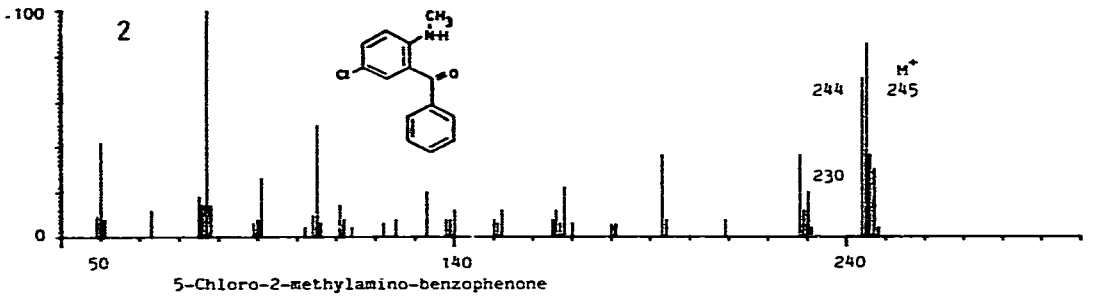
MONITORING PROGRAM FOR BENZODIAZEPINES AFTER HYDROLYSIS

Mass spectrum No.	Hydrolyzed drugs and metabolites	*m/e* (relative intensities in %)								Retention index
		211	230	241	244	249	262	276	285	
01	Bromazepam					100		28		2450
02	Camazepam		18		83					2100
02	Methyl-oxazepam		18		83					2100
03	Oxazepam		100							2050
03	Chlorazepate		100							2050
03	Chlordiazepoxide		100							2050
04	Clobazam		10				4	17		2225
05	Nor-			44	35					2210
06	Clonazepam	7	4	100				67		2470
07	(Acet-)amino-	72				4				2287
02	Diazepam		18		83					2100
03	Nor-/Oxazepam		100							2050
08	Flunitrazepam	22								2374
09	(Acet-)amino-	10	3		100					2796
10	(Acet-)amino-nor-	70	100							2167
11	Flurazepam									2554
12	Didesethyl-	100				4		16		2294
13	Hydroxyethyl-						100			2383
14	N-Desalkyl-		20			100				2031
15	Lorazepam		100							2180
16	Lormetazepam				100		17			2220
15	Nor-		100							2180
17	Medazepam				32					2236
18	Nor-		23							2278
03	Nor-diazepam		100							2050
19	Nitrazepam	4		88						2363
20	(Acet-)amino-	92								2225
03	Oxazepam		100							2050
21	Prazepam		17		14				65	2411
03	N-Desalkyl-		100							2050
22	Tetrazepam					55				2200
23	(Androsterone)		5	2	50			2		2476

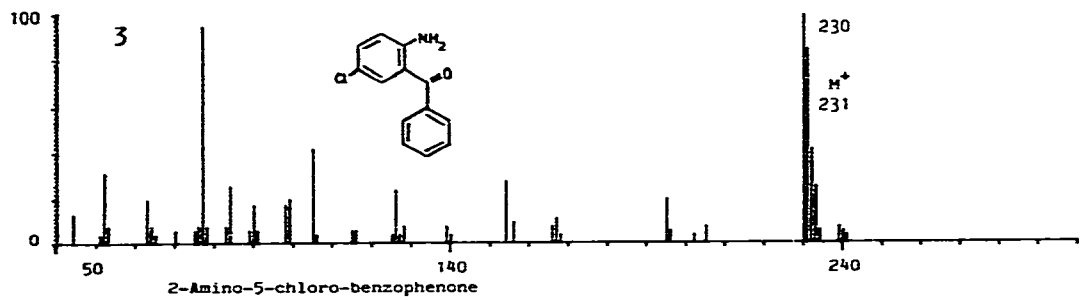
The entire mass spectra of the benzophenones are shown in Fig. 1 for the precise identification of the compounds. The retention indices were determined using a gas chromatograph combined with FID and N-FID with a temperature program. We found that the retention indices obtained with tempera-



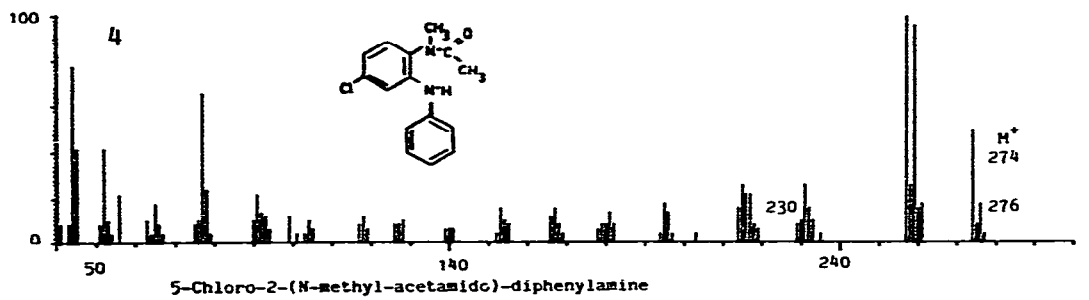
2-(2'-Amino-5'-bromo-benzoyl)-pyridine



5-Chloro-2-methylamino-benzophenone

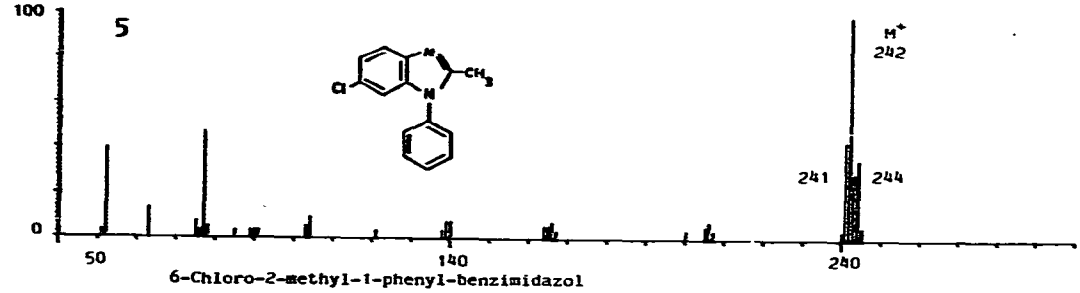


2-Amino-5-chloro-benzophenone

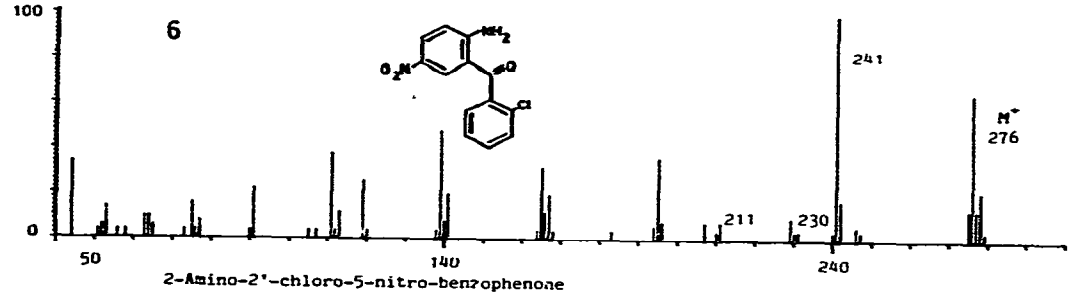


5-Chloro-2-(N-methyl-acetamido)-diphenylamine

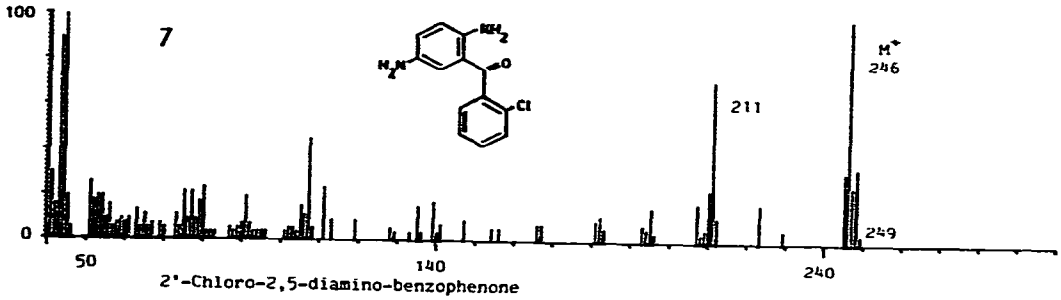
Fig. 1.



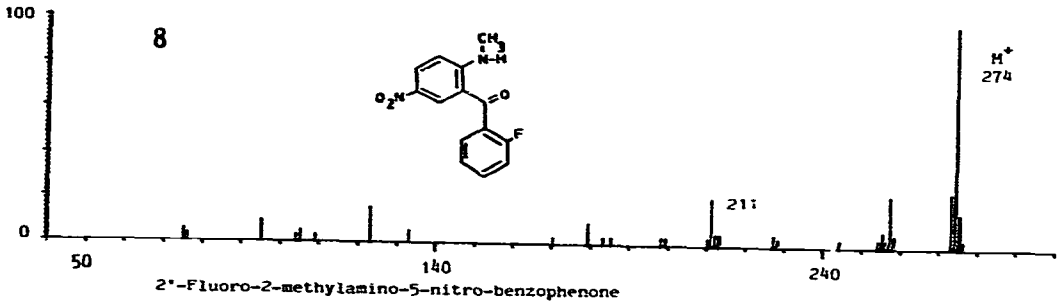
6-Chloro-2-methyl-1-phenyl-benzimidazol



2-Amino-2'-chloro-5-nitro-benzophenone



2'-Chloro-2,5-diamino-benzophenone



2'-Fluoro-2-methylamino-5-nitro-benzophenone

Fig. 1.

(Continued on p. 414)

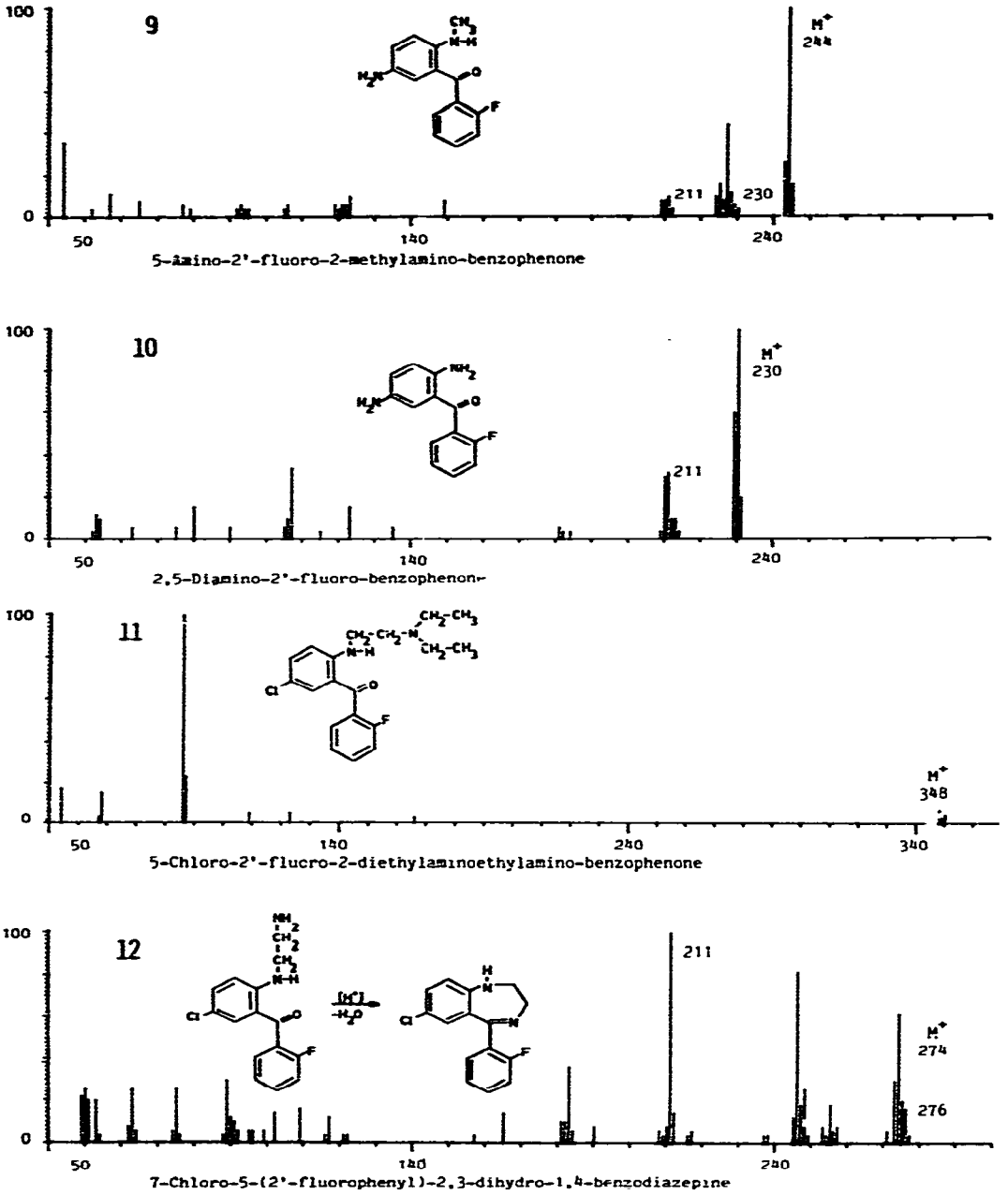
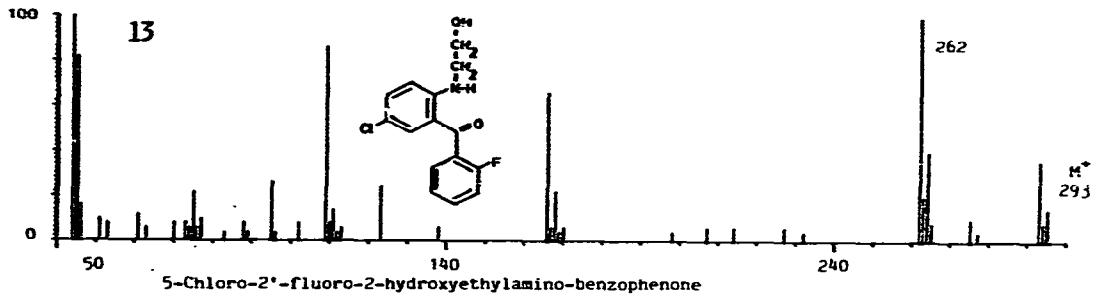
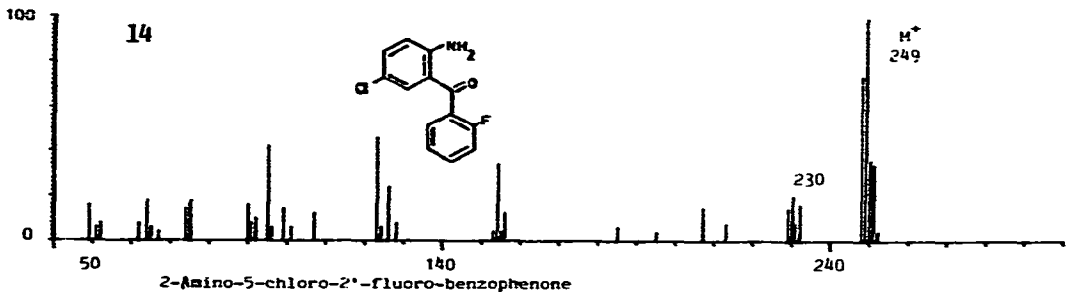

9
100
0
50
140
240
211
230
M+
244
5-Amino-2'-fluoro-2-methylamino-benzophenone
10
100
0
50
140
240
211
230
M+
2,5-Diamino-2'-fluoro-benzophenon-
11
100
0
50
140
240
340
M+
348
5-Chloro-2'-flucro-2-diethylaminoethylamino-benzophenone
12
100
0
50
140
240
211
M+
274
276
7-Chloro-5-(2'-fluorophenyl)-2,3-dihydro-1,4-benzodiazepine

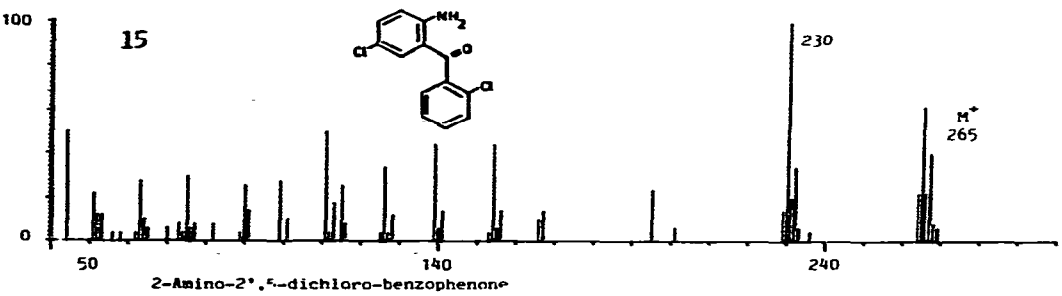
Fig. 1.



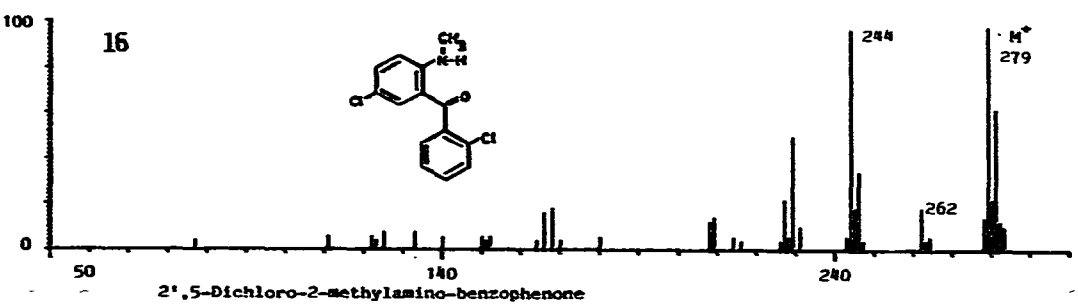
5-Chloro-2'-fluoro-2-hydroxyethylamino-benzophenone



2-Amino-5-chloro-2'-fluoro-benzophenone



2-Amino-2',5-dichloro-benzophenone



2',5-Dichloro-2-methylamino-benzophenone

Fig. 1.

(Continued on p. 416)

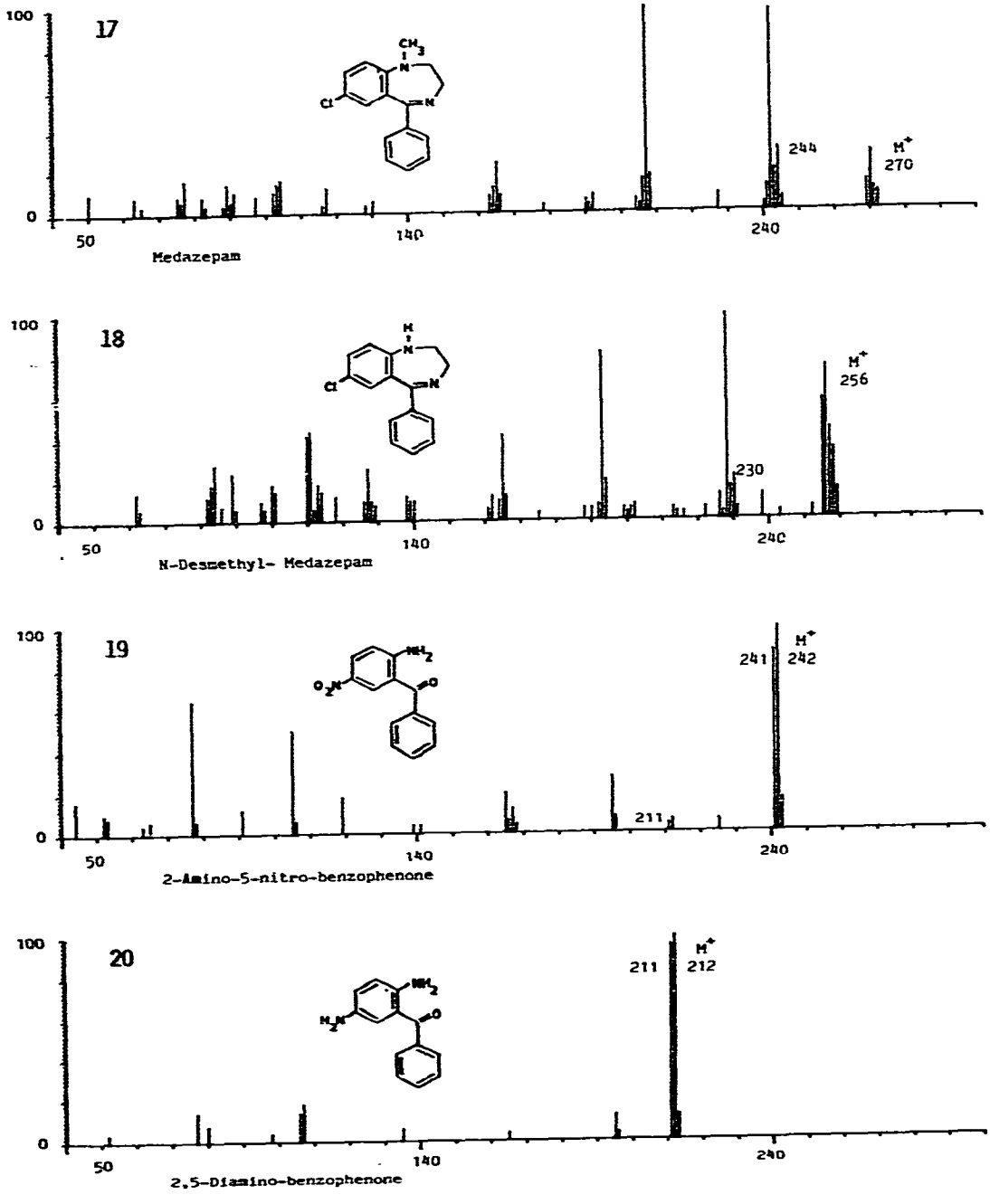

100
17
0
50
140
240
244
M+
270
Medazepam
100
18
0
50
140
240
230
M+
256
N-Desmethyl- Medazepam
100
19
0
50
140
240
241
M+
242
211
2-Amino-5-nitro-benzophenone
100
20
0
50
140
240
211
M+
212
2,5-Diamino-benzophenone

Fig. 1.

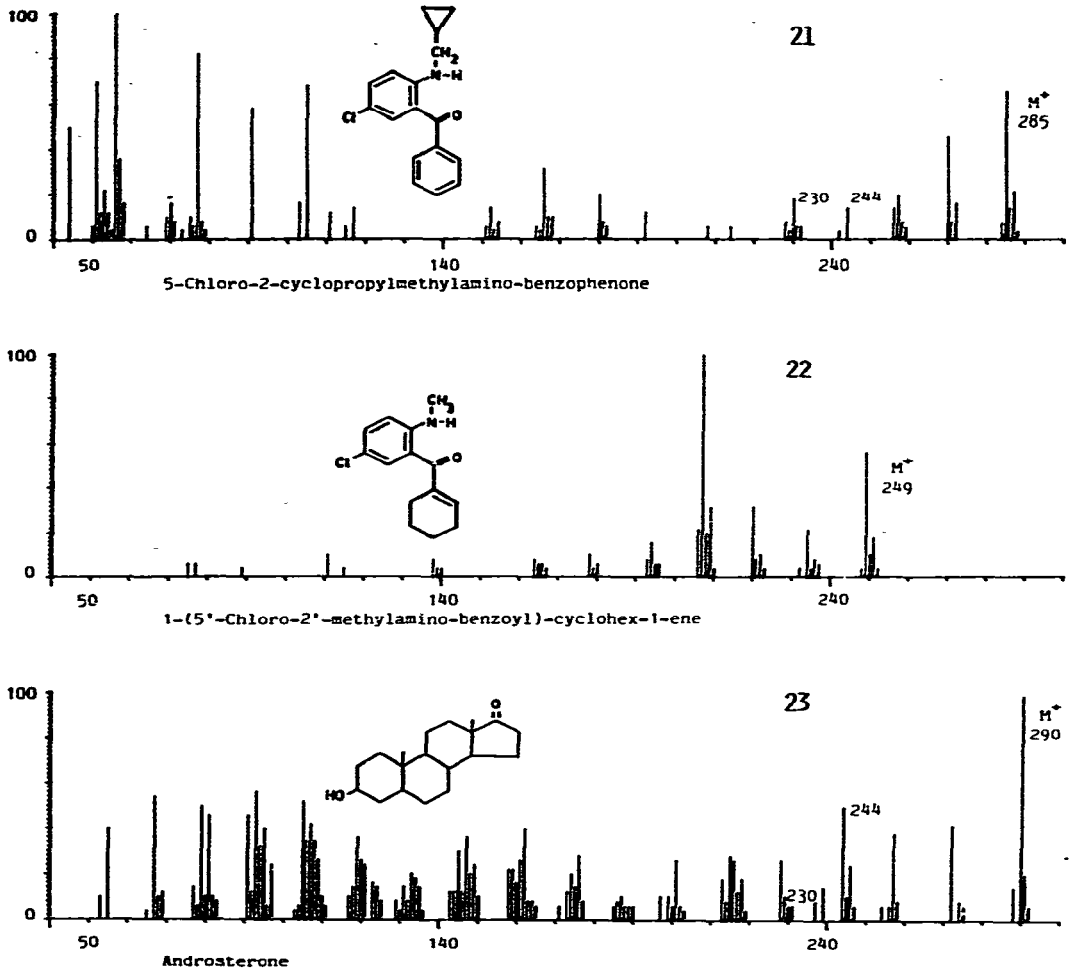


Fig. 1. Mass spectra of the hydrolysis products of benzodiazepines and androsterone.

ture programming were the same as those obtained using an isothermal procedure [6]. In our experience retention indices are not necessary when employing the GC—MS technique but may be useful to gas chromatographers without the latter facility and so they are given here. All investigations were carried out using the urine of man after therapeutic dosage with the exception of clonazepam, flunitrazepam and lormetazepam which were detected — in the absence of human samples — in the urine of rats.

Camazepam, clobazam, clonazepam, flurazepam and medazepam are almost completely excreted in the urine as their metabolites. Medazepam and its desmethyl metabolite are not hydrolyzable, because they contain no lactam ring.

In our experience androsterone is the only endogenous physiological substance that appears in the mass fragmentogram.

Although camazepam and diazepam and their metabolites are hydrolyzed to the same two benzophenones, ingestion of the two drugs can be differentiated

by the relative proportions of the two hydrolysis products. Thus, camazepam leads to more chloromethylaminobenzophenone (CMAB, from methyl-oxazepam) than aminochlorobenzophenone (ACB, from oxazepam), whilst diazepam leads to more ACB (from desmethyl-diazepam and oxazepam) than CMAB (from diazepam).

Only chlorazepate, chlordiazepoxide and oxazepam cannot be differentiated by the hydrolysis products because they and their metabolites are all hydrolyzed to ACB alone. If necessary, they can be identified afterwards in a second urine sample after enzymatic hydrolysis of the conjugates or in the blood.

For an illustration of the method a mass fragmentogram of a sample from a psychiatric clinic is shown in Fig. 2. The first peak at *m/e* 230 indicates ACB (mass spectrum No. 15) from lorazepam, and the peaks at *m/e* 230, 241 and and oxazepam, the peak at *m/e* 244 indicates CMAB (mass spectrum No. 2) from diazepam, the second peak at *m/e* 230 is aminodichlorobenzophenone (mass spectrum No. 15) from lorazepam, and the peaks at *m/e* 230, 241 and 244 indicate the physiological hormone androsterone (mass spectrum No. 23). This example shows that diazepam (in this case given therapeutically), its metabolites, and lorazepam (taken in abuse) can be precisely differentiated. This is impossible in such a short time with any method previously described in the literature.

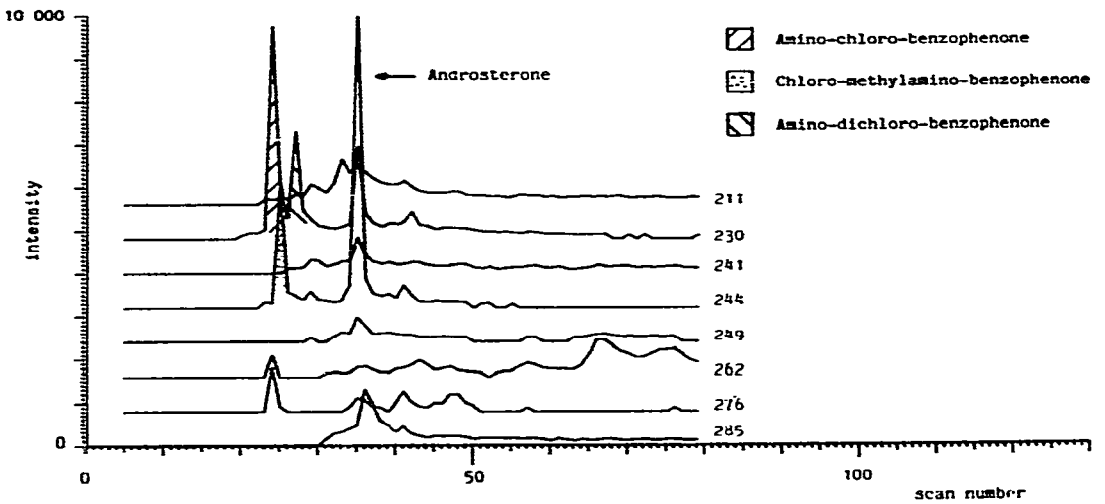


Fig. 2. Mass fragmentogram indicating different benzophenones from the urine of a benzodiazepine addict.

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